Exhibit 7

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MOLECULAR AND CELLULAR ASPECTS OF CARCINOGEN SCREENING TESTS

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METABOLISM OF *N*-NITROSODIMETHYLAMINE

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INTRODUCTION

It is now generally recognized that most carcinogenic chemicals exert their cancer-initiating effects only if they are converted by metabolic processes to chemically reactive species (Magee et al., 1975; Miller, 1978). The wide variety of chemical structures now known to be carcinogenic and the diverse group of enzymatic reactions leading to generation of ultimate carcinogens render it quite impossible to cover this area in a short review, except at a most superficial level. Therefore, in this article, only the metabolic activation of *N*-nitrosodimethylamine (NDMA) is discussed in detail.

This compound is a potent carcinogen (Magee et al., 1975, 1976), which has a simple chemical structure, is readily synthesized in a radioactively-labelled form and is metabolized relatively rapidly. It is, therefore, a useful research tool with which to study parameters of carcinogen uptake and metabolism and provides a model system from which some interesting conclusions can be drawn which may be applicable to other chemical carcinogens. A second reason for wishing to understand the 'pharmacokinetics' involved in the metabolic activation and clearance of NDMA is that there is direct evidence that humans are exposed to this carcinogen. It has been detected in many foods and can be formed by the reaction of nitrite with appropriate amines within the gastrointestinal tract (IARC, 1978). The compound has been detected in human blood after a meal (Fine et al., 1977; Lakritz et al., 1979), in human faeces (Wang et al., 1978) and in urine (Kakizoe et al., 1979). Although the levels measured in these experiments were quite low, they can be used to estimate exposures if appropriate calculations are made on the basis of the uptake and metabolism of the compound.

ABSORPTION AND UPTAKE OF NDMA

NDMA and *N*-nitrosodiethylamine (NDEA) are absorbed relatively slowly from the stomach, with a half-life of more than one hour (Hashimoto et al., 1976; Heading et al., 1974; Pegg, 1979). This uptake is, in fact, slower than the rate of metabolism of NDMA when small doses are administered to rats *via* a stomach tube (Diaz Gomez et al., 1977; Pegg, 1979). Uptake of the carcinogen is very rapid from the intestine and particularly from the upper part of the small intestine (Hashimoto et al., 1976; Heading et al., 1974; Pegg, 1979), where the half-life was only about three minutes. It appears, therefore, that absorption of these dialkylnitrosamines occurs predominantly from the duodenum when exposure is due to their presence in ingested food or by their formation by nitrosation at the acid pH of the stomach.

When NDMA is present in the blood, it readily passes into tissues. Autoradiographic studies in which ^{14}C-NDMA was administered to mice in the presence of an inhibitor of metabolism, to ensure that the carcinogen itself and not a metabolite was being investigated, have confirmed the original observation of Magee (reviewed in Magee & Barnes, 1967) that NDMA is distributed uniformly during a short period of exposure (Johansson & Tjälve, 1978). Although the question has not been addressed by any direct experimentation, it appears unlikely that any specific transport system is required for the uptake of NDMA. The compound appears to pass readily through cell membranes; therefore, all tissues in the body may contain it.

METABOLISM AND SITE OF TUMOUR PRODUCTION

Despite its ubiquitous distribution, NDMA produces tumours at only a few sites. In rats, large single doses (10–40 mg/kg) given by intraperitoneal injection produce kidney tumours (IARC, 1978; Magee et al., 1975, 1976). The incidence of kidney tumours can be increased to 100% by feeding a protein-free diet for seven days prior to administration of 60 mg/kg NDMA (McLean & Magee, 1970). Prolonged exposure of rats to much lower doses of the carcinogen, *via* the diet or drinking-water, results in the induction of liver tumours (IARC, 1978; Magee et al., 1976); dietary levels of 50 mg/kg (ppm) produce virtually 100% tumour incidence. NDEA also produces kidney tumours in rats when given in large single doses (280 mg/kg), and it results in liver tumours after prolonged feeding of much lower doses (IARC, 1978; Magee et al., 1976). Many other species have also been shown to be sensitive to the carcinogenic action of these compounds and to respond by development of tumours in the same organs. Certain other organs are also targets for tumour induction by NDMA and NDEA (for example, the oesophagus and the lung); but, in general, tumours are observed in only a very few sites, despite the widespread distribution of the carcinogens.

These results are in excellent agreement with the hypothesis that the carcinogenic action of NDMA is mediated *via* the formation of a chemically reactive species which is too unstable to escape from the cell in which it is generated. Tumours are observed only in those organs capable of metabolic activation. Metabolism of NDMA takes place predominantly in the liver, to a lesser extent in the kidney, and to a much smaller extent in the lung (Magee & Barnes, 1967; Magee et al., 1975, 1976; Montesano & Magee, 1974). The rate of metabolism is substantially greater than the rate of excretion, unless very large doses, exceeding the median lethal dose, are given (Heath, 1962). In some strains of rats, the ability of the liver to metabolize NDMA is reduced by feeding a diet deficient in protein, while that of the kidney is not (Swann & McLean, 1971; Waynforth et al., 1977); in rats fed such a diet, therefore, the kidney metabolizes a greater proportion of the dose than in controls, and the increased tumour incidence in the kidney is readily explained.

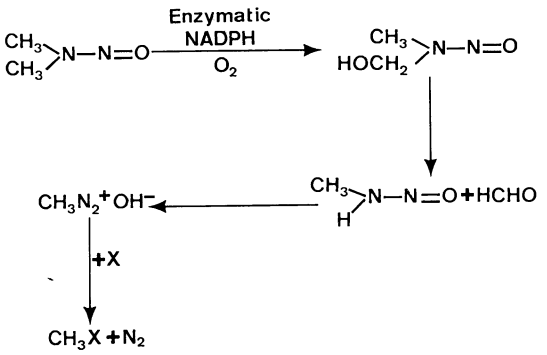
It should be noted that because NDMA metabolism is virtually complete and there is very little excretion of unchanged carcinogen, a moderate reduction in metabolism would not be expected to alter tumour initiation. Such inhibition of metabolism would be effective in altering carcinogenicity only if the rate were slowed sufficiently to allow for substantial elimination by excretion or by other pathways. This can be brought about by treatment with aminoacetonitrile, a powerful inhibitor of NDMA metabolism both *in vivo* and *in vitro* (Fiume et al., 1970; Hadjiolov & Mundt, 1974). Treatment with disulfiram prevented metabolism of NDEA and NDMA by the liver and reduced toxicity and hepatocarcinogenicity, but it also increased tumour incidence at other sites (Schmähl et al., 1976). These results all support the concept that metabolism to an alkylating species is the cause of toxicity and carcinogenicity. However, the finding that pregnenolone-16α-carbonitrile reduces toxicity without affecting alkylation of DNA or clearance of NDMA from the plasma has not yet been explained satisfactorily (Grandjean & Somogyi, 1976; Somogyi et al., 1972).

MECHANISM OF METABOLIC ACTIVATION

Metabolism of NDMA is thought to occur by means of the reaction scheme shown in Figure 1. Although there is good experimental evidence favouring this scheme, it has not been entirely proven. It is known that metabolism can be mediated by a liver microsomal fraction which requires NADPH and molecular oxygen (Magee & Barnes, 1967); it has therefore been postulated that the reaction involves oxidative demethylation to eliminate formaldehyde, which has been identified as a product. The resultant α-nitrosoaminocarbinol intermediate has never been identified; but it would be highly unstable, resulting in methylation of accessible nucleophiles, including nucleic acids, proteins and water, with formation of stoichiometric amounts of molecular nitrogen. An early study reported failure to find the expected release of nitrogen

FIG. 1. METABOLISM OF *N*-NITROSODIMETHYLAMINE

X represents a cellular nucleophile.



(Cottrell et al., 1977); however, recently, Milstein & Guttenplan (1979) have succeeded, using an experimental system in which considerable care was taken to remove endogenous nitrogen. The generation of nitrogen from NDMA metabolized by rats *in vivo* has also been found to be virtually stoichiometric with the degradation of the nitrosamine[1].

The postulated first step in the enzymatic decomposition of NDMA, outlined in Figure 1, is breakage of a carbon-hydrogen bond leading to an enzyme-bound carbanion, which would then be converted to the α-hydroxy derivative. Support for this mechanism derives from observations that fully deuterated NDMA reacts more slowly than NDMA itself (Dagani & Archer, 1976; Kroeger-Koepke & Michejda, 1979). This result is in accordance with the postulate that an initial enzymatic oxidation step is rate-limiting for the decomposition.

Further support for the idea that a hydroxymethyl derivative of NDMA is a reactive intermediate important in its carcinogenic action has been provided by studies of the carcinogenicity and chemical properties of methyl(acetoxymethyl)nitrosamine. This ester is converted to the presumed metabolite by the action of esterases (Kleihues et al., 1979; Roller et al., 1975; Wiessler & Schmähl, 1976); and the reactive alkylating species generated in this way produces a spectrum of alkylated products in DNA identical to those found when NDMA is administered to rats (Kleihues et al., 1979). The esterases capable of activating

[1] P.N. Magee, J. Holsman & D. Halliday, personal communication

methyl(acetoxymethyl)nitrosamine are much more widely distributed than the NDMA-activating system. Tumour induction by the methyl ester depends on the route of administration: intraperitoneal injection resulted in predominantly intestinal tumours, with some tumours in other abdominal viscera (Berman et al., 1979; Joshi et al., 1977; Ward et al., 1977); intravenous injection gave predominantly lung and heart tumours (Habs et al., 1978; Kleihues et al., 1979); and application *via* oral, subcutaneous or intrarectal routes produced tumours at the site of application (Habs et al., 1978; Wiessler & Schmähl, 1976). These results are readily explained by a rapid uptake of methyl(acetoxymethyl)nitrosamine into cells followed by rapid metabolism by esterases to the hydroxymethyl derivative, which decomposes spontaneously to alkylate tissue components in the cell. Because of the widespread distribution of the activating esterases and the rapid uptake into cells, the greatest degree of alkylation takes place in those organs near the site of application, or in those first exposed by uptake into the systemic circulation (Kleihues et al., 1979).

Decomposition of monomethylnitrosamine is thought to proceed *via* the formation of a methyldiazonium ion or a related species. The possible generation of diazomethane as an alkylating intermediate was ruled out by the demonstration that when d_6-NDMA was used, the resulting alkylated nucleic acids contained methyl groups that retained all three deuterium atoms (Lijinsky et al., 1968).

The overwhelming weight of experimental evidence suggests that the great majority, if not all, of NDMA metabolism occurs *via* the scheme shown in Figure 1. Further metabolism of the formaldehyde takes place to yield carbon dioxide. A substantial fraction of the methylating species derived from decomposition of the NDMA reacts with water, producing methanol, which is also oxidized to carbon dioxide. The $^{14}CO_2$ in the expired air following administration of ^{14}C-NDMA (Heath, 1962; Fiume et al., 1970) or in the gaseous phase of incubations of tissue slices (Montesano & Magee, 1974; Swann & McLean, 1971) can be quantitated, and these measurements can provide some information concerning the rate of metabolism of the carcinogen; however, account must be taken of the lag time before conversion of the formaldehyde or methanol to $^{14}CO_2$, owing to the other oxidative steps involved. The rate-limiting step in $^{14}CO_2$-production may not be the metabolism of the nitrosamine (Den Engelse et al., 1975).

The almost quantitative release of nitrogen, the substantial production of $^{14}CO_2$ and the high activity of the liver enzymes catalysing the oxidative demethylation suggest that other metabolic pathways are likely to account, at best, for only a very small fraction of the total metabolism. It has been reported, however, that a liver enzyme system, prepared by precipitation at pH5, catalysed production of *N*,*N*-dimethylhydrazine and that microsomal fractions produced methylamine and *N*-methylhydrazine (Grilli & Prodi, 1975). The extent to which such a reaction occurs *in vivo* is likely to be small, as argued above; but its importance cannot be overlooked, and confirmation and extension of these results is highly desirable.

ENZYMOLOGY OF ACTIVATION

Many reports have attested to the presence of enzymes that catalyse the oxidative demethylation of NDMA in rodent liver extracts. Much confusion was generated in this field by the publication of apparently contradictory results in which inducers of hepatic microsomal enzymes were claimed to both increase and inhibit NDMA demethylase activity. This discrepancy has now been explained by work in a number of laboratories, which shows that there are two or more enzyme forms that catalyse this reaction (Arcos et al., 1977; Kroeger-Koepke & Michejda, 1979; Lake et al., 1976; Lotlikar et al., 1978; Sipes et al., 1978). These forms have different kinetic properties with respect to the nitrosamine substrate concentration; analysis of the kinetics suggests that one form has a K_m of substantially less than 1 mM whereas the others have K_m values of the order of 100 mM.

Since the median lethal dose of NDMA is such that a concentration of more than 1 mM is unlikely to be reached if the compound is distributed uniformly (and, as discussed above, there is autoradiographic evidence for such uniform distribution), it is unlikely that the high-K_m forms of the enzyme play a significant role in the metabolism of NDMA under experimental pathophysiological conditions. Therefore, although it has been shown that increases in this activity take place after rats, mice, hamsters and guineapigs are treated with inducers of drug metabolism, such as Aroclor 1254, 3-methylcholanthrene and phenobarbital (Arcos et al., 1977; Czygan et al., 1973; Guttenplan & Garro, 1977; Lake et al., 1974; Lotlikar et al., 1978; Sipes et al., 1978), such increases are not likely to be reflected in an increased rate of metabolism of NDMA *in vivo*. Indeed, the low-K_m form of the enzyme is actually repressed by Aroclor 1254, phenobarbital, 3-methylcholanthrene, pregnenolone-16α-carbonitrile and β-naphthoflavone in some species (Arcos et al., 1975, 1977; Lotlikar et al., 1978; Sipes et al., 1978); and under some conditions these agents actually protect against the carcinogenic or toxic effects of NDMA (Argus et al., 1978).

These recent studies have, therefore, substantially clarified the situation and emphasize the importance of making *in vitro* measurements at physiologically relevant concentrations of substrate and of restricting comparison between different experimental results to those obtained under identical assay conditions. At present, however, little is known of the mechanism by which induction or repression of the various forms of the NDMA demethylase is brought about or of the relationship between the various forms. It should also be noted that there are striking species and strain differences in the response of the enzyme(s) to inducers/repressors (Arcos et al., 1977; Kroeger-Koepke & Michejda, 1979; Lotlikar et al., 1978).

These questions are unlikely to be answered satisfactorily until highly purified preparations of the enzyme are available; and, despite

much effort, this has not been achieved. Indeed, there is still disagreement as to the role of the cytochrome P-450-dependent mixed-function oxidase in the metabolism of NDMA. On the one hand, both the low- and high-K_m forms of the enzyme are microsomal, require NADPH and oxygen and are inhibited by carbon monoxide or pretreatment with cobalt chloride. Reconstitution studies with partially purified extracts revealed a need for both cytochrome P-450 and NADPH-cytochrome c reductase (Lotlikar et al., 1975). On the other hand, the atypical repression of the low-K_m form by compounds considered to be inducers of microsomal mixed-function oxidases, the relatively weak inhibition of activity by well known inhibitors of these oxidases, such as SKF 525A (Godoy et al., 1978; Lake et al., 1976), the greater stability under storage of NDMA demethylase compared with cytochrome P-450 and other mixed-function oxidase enzyme activities in liver extracts (Lake et al., 1976) and the inhibition of NDMA metabolism by a variety of compounds that do not inhibit other mixed-function oxidases (Lake et al., 1976; Phillips et al., 1977) suggest that NDMA metabolism does not occur *via* a typical mixed-function oxidase. There is also evidence that, although the NDMA demethylase system is microsomal, its activity is stimulated by a cytosolic component (Godoy et al., 1978; Kroeger-Koepke & Michejda, 1979; Lake et al., 1976; Magee & Barnes, 1967). It seems probable, therefore, that the NDMA demethylase is a complex multi-component system which differs significantly from the 'typical' mixed-function oxidases. There is much evidence to show that there are multiple cytochrome P-450 species with different substrate specificities; if only one such species is involved in NDMA metabolism, the lack of correlation with measurements based on overall, total cytochrome P-450 can readily be explained.

METABOLISM OF NDMA TO MUTAGENIC OR CARCINOGENIC AGENTS *IN VITRO*

Isolated hepatocytes have been shown to be transformed by exposure to NDMA (Montesano et al., 1975; Williams, 1976), and mutations can be obtained in response to the carcinogen when various test cells are co-cultivated with liver cells (Langenbach et al., 1978). Initiation of DNA repair is also produced by exposure of isolated hepatocytes to NDMA (Williams & Laspia, 1979). These results suggest that metabolic activation is taking place in such cells, but, since the NDMA concentrations used in these experiments were relatively high, it is likely that the high-K_m forms of the NDMA demethylase are responsible for the metabolism; therefore, the relevance of the low-K_m form to *in vivo* metabolism is not tested in these experiments. Similarly, as pointed out by Argus & Arcos (1978), the production of mutagenic metabolites by liver microsomes has been observed by numerous groups (Bartsch et al., 1975; Czygan et al., 1973; Frantz & Malling, 1975; Kuroki et al., 1977); but they have all used such high concentrations of NDMA that the physiologically relevant metabolism was not tested. Very recently, Guttenplan (1979) has reported the induction of mutations in *Salmonella typhimurium* with

lower concentrations of NDMA in the presence of microsomes from untreated mice.

REACTION WITH NUCLEIC ACIDS

The methylating agent derived from NDMA reacts with cellular macromolecules, including protein, RNA and DNA. Extensive and comprehensive reviews describe the sites and extents of such methylation (Lawley, 1976; Magee & Barnes, 1967; Magee et al., 1976; Pegg, 1977a; Singer, 1977). Particular attention has been paid to the reaction with DNA, since it is widely believed that alterations to DNA may provide the basis of tumour initiation. It has been known for some time that the major product of this reaction is 7-methylguanine, but a wide variety of other products are now recognized, including methylphosphate triesters, 1-, 3- and 7-methyladenine, 3- and O^6-methylguanine, 3- and O^4-methylcytosine and 3-, O^2- and O^4-methylthymine. Analagous products have been reported to be present in RNA. In livers of rats treated with NDMA, the extent of alkylation of cytoplasmic RNA or of mitochondrial DNA was slightly higher than that of nuclear DNA (Swann & Magee, 1968; Wilkinson et al., 1975), but the difference was only small. In view of the high reactivity of the methylating species generated by metabolism of NDMA, it is perhaps surprising that it can travel from a microsomal site of generation to the nucleus in order to react with DNA. An explanation of this phenomenon may be found in a recent preliminary report which suggests that isolated nuclei are capable of metabolic activation of NDMA (Grandjean et al., 1978). Similar nuclear activation has now been firmly established for several other carcinogens (Bresnick et al., 1977; Kawajiri et al., 1979; Stout & Becker, 1979). It is, therefore, possible that most of the reaction with DNA is mediated *via* the nuclear activation pathway, even though the greater part of the carcinogen is metabolized by the microsomal system.

It is possible to measure the extent to which a chemical carcinogen is metabolized by a particular organ by determining the amount of a product formed within the tissue. This is a particularly valuable method for comparing the degree of metabolism within different organs, since the reactive ultimate carcinogen is too unstable to react significantly in organs other than those in which it was generated. It is necessary that the product measured be sufficiently stable (or at least equally persistent in all organs, and its persistence independent of the amount of product) that the content measured not be affected significantly by its loss during the period of metabolism. Thus, of the alkylated bases produced by dialkylnitrosamines, 7-alkylguanine in DNA is particularly suitable for this purpose, because its rate of loss is slow (half-life, more than fifty hours) and independent of the extent of alkylation or the type of tissue, provided that allowance is made for the rate of cell division (Pegg, 1977b; Pegg & Hui, 1978a). It also has the advantage that analytical measurement is relatively simple (Pegg, 1977a; Swann & Magee, 1968). In contrast, the alkylated adenine derivatives are lost too quickly from DNA; O^6-alkylguanine

is lost at a different rate depending on the tissue or the extent of reaction; and the pyrimidine and alkylphosphate triesters are more difficult to measure (Pegg, 1977a; Pegg & Hui, 1978b; Singer, 1977).

Table 1 shows the amounts of 7- and O^6-alkylguanine present in rat liver DNA at various times after administration of doses of NDMA and NDEA.

Table 1. Alkylated guanine derivatives present in rat liver DNA after administration of *N*-nitrosodimethylamine (NDMA) or *N*-nitrosodiethylamine (NDEA)

Nitrosamine	Dose (mg/kg)	Time (hrs)	Alkylated guanines in DNA (μmol/mol guanine)	
			7-alkylguanine	O^6-alkylguanine
NDMA	0.01	0.5	3.2	0.25
	0.01	2.0	3.3	0.07
	0.02	0.5	5.3	0.41
	0.02	2.0	5.4	0.10
	0.02	4.0	4.6	0.04
	0.04	0.5	12.5	0.64
	0.04	2	10.8	0.36
	0.04	4	11.0	0.29
NDEA	0.5	0.5	2.5	1.4
	0.5	2	2.7	1.0
	0.5	4	2.6	0.6
	2.0	1.5	9.6	4.7
	2.0	3.75	9.7	2.8
	2.0	6.0	7.9	2.2
	4.0	2	18.8	10.0
	4.0	5	19.8	7.7
	4.0	8	15.8	4.7

It can be seen that the reaction with DNA, as measured by production of 7-alkylguanine, is complete within thirty minutes after doses of 10-40 μg/kg NDMA. The content of 7-methylguanine did not change over a further 3.5-hour period, but that of O^6-methylguanine decreased by more than 65%. Similarly, after doses of 0.5-4 mg/kg of NDEA, 7-ethylguanine levels were maximal at the earliest times measured (30 min to 2 hrs) and declined only slowly, whereas O^6-ethylguanine levels were reduced much more rapidly. The relative proportions of the alkylated bases produced initially in DNA by any particular alkylating species should be constant and irrespective of the dose, and this has generally been found

to be the case. For instance, the ratio of O^6-:7-methylguanine produced by NDMA is about 0.115 (Pegg, 1977a,b; Pegg & Hui, 1978a), whereas the ratio of O^6-:7-ethylguanine produced by NDEA is about 0.7 (Pegg & Balog, 1979). It has been suggested by Scherer et al. (1977) that this is not invariably the case and that a lower ratio was observed following small doses of NDEA. However, as shown in Table 1 and discussed in more detail elsewhere (Pegg & Balog, 1979), the lower ratio is caused by more efficient removal of the O^6-ethylguanine from DNA following alkylation to only small extents by low doses of NDEA.

A final advantage to measuring the appearance of reaction products rather than loss of the carcinogen is that the former method provides information concerning the nature of the ultimate carcinogen derived by metabolic activation. The spectrum of methylated bases produced in DNA by NDMA *in vivo* is exactly the same as that produced by reaction with the methylating species derived by chemical decomposition of *N*-methyl-*N*-nitrosourea *in vitro*, both in the sites alkylated and the relative proportions of alkylated products (Pegg, 1977a; Singer, 1977). This provides some assurance that the reactive metabolite is the methyldiazonium ion or an equivalent species, as described above. The simple chemical structure of NDMA renders it improbable that any reactive species other than a methylating agent could be the ultimate carcinogen; however, with other, more complex chemicals it is likely that more than one reactive species may be generated by metabolic reaction. Which of these species is responsible for the reaction with DNA may be determined by the subcellular generation sites, reaction with competing nucleophiles and abilities to serve as substrate for further metabolism. Only by measurement of the product of reaction with the target molecule can the identity of the ultimate carcinogen be established completely, and it follows from this that one cannot be certain of the ultimate carcinogen without knowing the target molecule.

CLEARANCE OF NDMA

Several workers have measured the rate of loss of administered doses of NDMA or NDEA from the blood in rats (Heath, 1962; Swann & McLean, 1971; Wishnok et al., 1978). In experiments with relatively large doses of NDMA (50 mg/kg), the concentration in the blood fell linearly with time at a rate corresponding to the metabolism of 4.9-5.7 mg/hr per kg body weight in rats fed a control diet, and this decreased to 2.7 mg/hr in rats maintained on a protein-free diet (Heath, 1962; Swann & McLean, 1971). Similarly, after doses of 200 mg/kg NDEA, it was decomposed at a rate of about 9 mg/hr per kg body weight (Heath, 1962). These results show that the rate of metabolism of these doses of the nitrosamine was not influenced by the concentration and that the metabolic system was effectively saturated over the time during which measurements were made. However, measurements were not continued over the entire time course of dialkylnitrosamine metabolism, and these values cannot be used for calculating clearance rates of much lower doses of the carcinogens.

It has been claimed that after doses of 3 mg/kg NDMA and 25 mg/kg NDEA, the disappearance of the carcinogens from the blood followed first-order kinetics,with a rate constant of about 0.018 min^{-1} in control rats and 0.013 in rats fed a lipotrope-deficient diet (Wishnok et al., 1978). Careful consideration of the data presented in this paper reveals very large standard errors and significant deviation from first-order kinetics, rendering interpretation difficult. There are, therefore, no reliable results concerning the true rates of metabolism of low doses of dialkylnitrosamine, approximating those to which the general population may be exposed. However, as shown in Table 1, measurements of 7-alkylguanine present in liver DNA after intraperitoneal administration of 10-40 µg/kg NDMA or 0.5-4 mg/kg NDEA show that reaction is complete within thirty minutes or at most two hours, respectively. These results and those described below, in which oral administration of NDMA was studied, indicate that small doses of the nitrosamines may be metabolized very rapidly. Such rapid clearance would lead to a substantial underestimation of exposure to dialkylnitrosamine if estimates were based only on the steady-state blood level, with no correction for clearance rates.

IMPORTANCE OF ROUTE OF ADMINISTRATION TO SITE OF METABOLISM

When NDMA was administered to rats by intraperitoneal, intravenous or oral administration at doses of 1 mg/kg or more, the ratio of alkylation of DNA in the liver to that in the kidney, as measured by 7-methylguanine production, was approximately the same (Diaz Gomez et al., 1977; Pegg, 1977a, 1979; Pegg & Hui, 1978a; Swann & Magee, 1968). This ratio was about 5-10, depending on the experimental conditions, and is in agreement with results of *in vitro* studies in which the capacity of liver slices to metabolize NDMA was about eight times greater than that of kidney slices (Montesano & Magee, 1974; Swann & McLean, 1971). The same ratio was observed when lower doses of NDMA (1-250 µg/kg) were given by intravenous injection (Pegg, 1979; Table 2). However, as previously reported by Diaz Gomez et al. (1977), when such low doses of NDMA were given by oral administration, the ratio of reaction with the liver to that with the kidney was much higher (Pegg, 1979; Table 2). The increase in the ratio was due to a marked decline in the proportion of the carcinogen metabolized by the kidney. The alkylation of liver DNA is proportional to dose over the range 1 µg/kg-20 mg/kg and is not greatly affected by the route of administration. Alkylation of kidney DNA, however, is much less after low doses when given by oral administration than when given by intravenous or intraperitoneal injection (Diaz Gomez et al., 1977; Pegg, 1977b, 1979; Pegg & Hui, 1978a).

The most probable explanation of these results is that NDMA given by oral administration is absorbed rapidly from the upper part of the small intestine into the portal blood supply and is then metabolized by the liver. Provided that the dose is sufficiently low, the liver metabolizes virtually all of the carcinogen in a 'first pass' clearance

Table 2. Effect of route of administration of *N*-nitrosodimethylamine on alkylation of liver and kidney DNA 6 hrs later

Route of administration	Dose (μg/kg)	Alkylation of DNA (μmol 7-methylguanine/mol guanine)		Ratio
		Liver	Kidney	
Oral	1	0.48	0.01	48
I.v.	1	0.40	0.06	6.7
Oral	50	21	0.4	52
I.v.	50	24	2.6	9.2
Oral	100	49	1.5	33
I.v.	100	45	5.2	8.7

effect well known from the pharmacology of many other drugs. The liver thus prevents the carcinogen from interacting with other organs such as the kidney. This hepatic clearance of NDMA has a number of important consequences.

Firstly, there is now considerable evidence that a critical alkylation product that leads to tumour initiation by simple *N*-nitroso compounds is O^6-alkylguanine and that certain organs are more resistant than others to carcinogenesis by these agents because they have a greater capacity to remove O^6-alkylguanine from their DNA (Goth & Rajewsky, 1974; Pegg, 1977a; Singer, 1979): the liver has the greatest ability to catalyse this removal reaction (Pegg, 1977a,b, 1978; Pegg & Hui, 1978a). After small oral doses of NDMA, therefore, virtually all of the reaction is with the organ best able to remove a critical alkylation product. As described above, continued feeding of NDMA produces tumours in the liver, whereas large single doses induce renal tumours. A contributing factor to the absence of kidney tumours in rats fed NDMA may be the low degree of interaction with this organ unless doses sufficiently high to avoid clearance by the liver are given. It is important to note that the presence of an active enzyme system that removes O^6-methylguanine from liver DNA in rat liver does not imply the existence of a threshold dose below which no significant carcinogenic effect would be expected. It merely implies that at some point in the dose-response curve the risk of tumours developing increases more rapidly than in proportion to the dose rate. In fact, this phenomenon has been observed in long-term feeding experiments with NDMA in which liver tumours were produced in rats[1] (IARC, 1978).

[1] R. Peto, personal communication

Secondly, the extent to which the liver might act to prevent interaction of the carcinogen with other organs may depend on several physiological parameters. For example, when the ability of the liver to metabolize NDMA is impaired (e.g., by feeding a protein-deficient diet, as discussed above), a greater fraction of the carcinogen may become available for reaction with other organs. Also, since uptake of NDMA is much more rapid from the small intestine than from the stomach, agents that retard gastric emptying might be expected to slow the rate of absorption. Agrelo et al. (1978) have recently published data which show that the presence of fat retards the rate of uptake and the metabolism of oral doses of NDMA. It is possible that reduction of the rate of absorption of the carcinogen in this way may increase its clearance by the liver, since the lower concentration in the portal blood that would result would give the hepatic enzymes more chance to effect complete metabolism.

Finally, it has been reported that NDMA and NDEA were present in human peripheral blood samples, and that the amounts increased after a meal (Fine et al., 1977; Lakritz et al., 1979). Calculations of total daily exposures have been made on the basis of these figures (National Research Council, 1978), but without knowledge of the clearance rate these calculations may be seriously in error and may underestimate total exposure. The results with rats suggest that orally ingested dialkylnitrosamines would appear in the systemic circulation only when the capacity of the liver to metabolize them in a first-pass effect is exceeded. Since rat and human liver slices have comparable levels of metabolizing activity (Montesano & Magee, 1974), the presence in peripheral blood of dialkylnitrosamines derived from the gastrointestinal tract may indicate a substantially greater exposure.

SUMMARY

NDMA and NDEA are metabolized by a microsomal enzyme system that requires NADPH and oxygen. This metabolism leads to an unstable product which decomposes to yield a reactive alkylating species. This species is too reactive chemically to influence significantly organs other than those in which it was generated. Alkylation of cellular components, particularly DNA, is a critical event in the initiation of tumours by these carcinogens. The greatest capacity to metabolize these nitrosamines to alkylating agents is found in the liver, but other organs, including the oesophagus, lung and kidney, are also capable of activation. These organs may be more susceptible to alkylation than the liver because they have a lesser ability to catalyse the removal of O^6-alkylguanine from their DNA. However, orally administered doses of NDMA and the NDMA formed by nitrosation reactions

within the gastrointestinal tract are rapidly absorbed from the upper part of the small intestine and carried to the liver in the portal blood supply. When small doses are given in this way, the capacity of the liver to metabolize the carcinogen is sufficient that the nitrosamine is effectively cleared in a 'first-pass' effect, leaving very little to interact with other organs. This has two important consequences: firstly, levels of NDMA found in peripheral blood may be significantly lower than those expected on the basis of total dietary exposure because of the rapid metabolism and effective clearance of the carcinogen by the liver; secondly, physiological factors leading to reduction of the metabolic activation in the liver may result in more of the carcinogen being metabolized by other tissues and in a greater risk of cancer developing in those tissues.

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